Molecular Characterization of Iodotyrosine Dehalogenase Deficiency in Patients with Hypothyroidism

Gijs Afink, Willem Kulik, Henk Overmars, Janine de Randamie, Truus Veenboer, Arno van Cruchten, Margarita Craen, and Carrie Ris-Stalpers

Laboratories of Pediatric Endocrinology (G.A., J.d.R., T.V., C.R.-S.) and Genetic Metabolic Diseases (W.K., H.O., A.v.C.), Academic Medical Center, University of Amsterdam, 1100 DD Amsterdam, The Netherlands; and Department of Pediatric Endocrinology (M.C.), Ghent University Hospital, B-9000 Ghent, Belgium

Context: The recent cloning of the human iodotyrosine deiodinase (IYD) gene enables the investigation of iodotyrosine dehalogenase deficiency, a form a primary hypothyroidism resulting from iodine wasting, at the molecular level.

Objective: In the current study, we identify the genetic basis of dehalogenase deficiency in a consanguineous family.

Results: Using HPLC tandem mass spectrometry, we developed a rapid, selective, and sensitive assay to detect 3-monoiodo-L-tyrosine and 3,5-diodo-L-tyrosine in urine and cell culture medium. Two subjects from a presumed dehalogenase-deficient family showed elevated urinary 3-monoiodo-L-tyrosine and 3,5-diodo-L-tyrosine levels compared with 57 normal subjects without thyroid disease. Subsequent analysis of *IYD* revealed a homozygous missense mutation in exon 4 (c.658G>A p.Ala220Thr) that co-segregates with the clinical phenotype in the family. Functional characterization of the mutant iodotyrosine dehalogenase protein showed that the mutation completely abolishes dehalogenase enzymatic activity. One of the heterozygous carriers for the inactivating mutation recently presented with overt hypothyroidism indicating dominant inheritance with incomplete penetration. Screening of 100 control alleles identified one allele positive for this mutation, suggesting that the c.658G>A nucleotide substitution might be a functional single nucleotide polymorphism.

Conclusions: This study describes a functional mutation within *IYD*, demonstrating the molecular basis of the iodine wasting form of congenital hypothyroidism. This familial genetic defect shows a dominant pattern of inheritance with incomplete penetration. **(*J Clin Endocrinol Metab* 93: 4894–4901, 2008)**

The cascade of events leading to the production of T_4 and T_3 in the thyroid is well documented (1). A defect in this cascade may lead to insufficient thyroid hormone production, and causative mutations in numerous genes explaining the molecular cause have been described (2–7). The concerted actions of thyroid peroxide and dual oxidase 2 result in the iodination of specific 3-monoiodo-L-tyrosine (MIT) and 3,5-diodo-L-tyrosine (DIT) within thyroglobulin (TG). The subsequent coupling of MIT and DIT residues generates T_4 and T_3 that are secreted into the circulation. Upon degradation of TG within the thyrocyte, noncoupled MIT and DIT are also released. Because these iodotyrosines contain the major amount of iodide within TG, recycling of these compounds is essential to salvage the rare element iodide.

For decades the importance of this process for the maintenance of euthyroidism, especially in iodine-deficient areas, has

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Abbreviations: CV, Coefficient of variation; DD, dehalogenase deficiency; DIT, 3,5-diodo-L-tyrosine; fT_4, free T_4; HPLC-MS/MS, HPLC tandem mass spectrometry; IS, internal standard; IYD, iodotyrosine deiodinase; LOD, limit of detection; MIT, 3-monoiodo-L-tyrosine; MRM, multiple reaction monitoring; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; S/N, signal-to-noise ratio; TG, thyroglobulin.

been well accepted (8), and patients with iodotyrosine dehalogenase deficiency (DD) (Online Mendelian Inheritance in Man 274800) were already described in the 1950s based on chromatographic studies with radioactive-labeled compounds and measurement of the enzymatic activity in goitrous thyroid gland (9, 10). In general these patients will develop goiter when dietary iodide is limiting, are (compensated) hypothyroid, have a rapid and high uptake of radioiodine, and secrete higher than normal MIT and DIT in their urine (6). However, final diagnosis of these patients was not supported by molecular evidence because the causal gene had not been cloned.

We (11), and others (12, 13) have recently identified the gene iodotyrosine deiodinase (*IYD*) (also known as *Dehal1* or *C6orf71*) encoding the enzyme responsible for MIT and DIT deiodination. The IYD protein belongs to the nicotinamide adenine dinucleotide (NADH) oxidase/flavin reductase superfamily, its sequence is highly conserved among mammals, and has a high level of similarity to bacterial NADH oxidase/flavin reductases (12). IYD is mainly expressed in the thyroid, but IYD mRNA is also detected in the liver, kidney, and colon (11, 13). The identification of IYD has provided detailed biochemical data on the *in vitro* deiodination of MIT and DIT. Recently, the first homozygous mutations in the IYD gene have been reported in four hypothyroid patients included for genetic analysis because of biochemical or clinical features suggestive of DD (14).

In the current study, we describe a rapid, highly selective, and sensitive HPLC tandem mass spectrometry (HPLC-MS/MS) method for the detection of MIT and DIT that facilitates the detection of these metabolites in urine and enables structure-function analysis of *in vitro*-produced wild-type and mutant protein.

Application of the assay identified two patients with congenital hypothyroidism that displayed markedly increased urinary DIT and MIT levels. Both individuals belong to a family with a putative DD, and we identified a causal missense mutation within *IYD* that completely abolishes enzymatic activity. The phenotype displays dominant inheritance in a heterozygous sib who presented with nonautoimmune goitrous hypothyroidism at the age of 15 yr.

Materials and Methods

MIT-DIT assay

Reagents

Analytical grade solvents were purchased from Merck KGaA (Darmstadt, Germany), and MIT, DIT, and 3-chloro-L-tyrosine (MCT) from Sigma Chemical Co. (St. Louis, MO). Other chemicals were of the highest purity available. Butanolic HCl (3 M) was prepared from butanol and acetyl chloride (4:1).

Internal standard (IS) and calibrators

MIT and DIT stock solutions (0.5 μM) and MCT (IS) stock solution (1.6 μM) were prepared in distilled water or pooled urine. Three calibrator sets were prepared by adding 60 μl IS and a range of MIT and DIT stock solution volumes to 100 μl of: 1) water, 2) pooled urine, or 3) cell culture medium.

Sample preparation for assay validation. To 100 μl urine sample, 60 μl IS solution and 60 μl MIT and DIT stock solution (dissolved in either water or pooled urine) were added. After vortexing, samples were evaporated to dryness (N_2, 40C), derivatized with butanolic HCl (60 C, 15 min) to improve the signal-to-noise ratio (S/N), followed by evaporation to dryness, and subsequently reconstituted in 100 μl 2% acetonitrile in water. After determination of initial creatinine values by colorimetric Jaffe assay, all urine samples were adjusted to a final creatinine concentration between 0.1 and 1 μM.

Sample measurements

To each 100 μl urine sample, 60 μl IS solution was added. After vortexing, samples were evaporated to dryness (N_2, 40C), derivatized

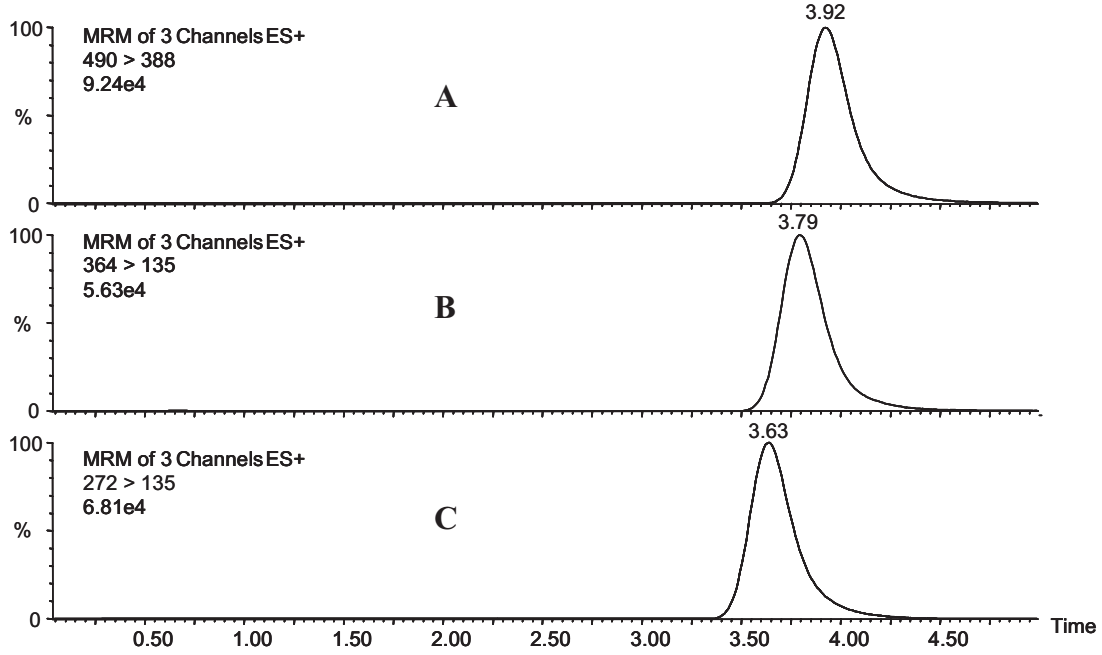


FIG. 1. MRM chromatogram of DIT (trace A), MIT (trace B), and MCT (trace C).

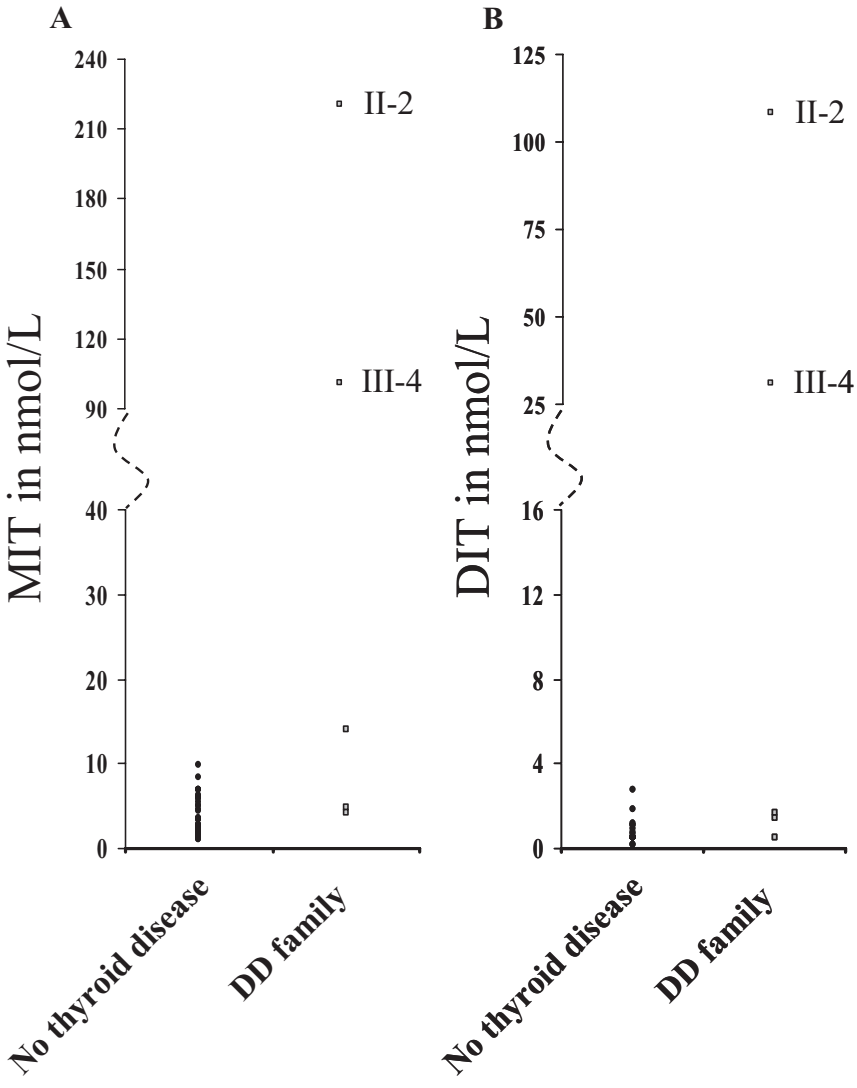


FIG. 2. MIT (A) and DIT (B) concentrations in human urine samples were determined by HPLC-MS/MS. Samples were from apparently healthy controls (No thyroid disease). In addition, five members of a family with dehalogenase defect (DD family) were included as a separate group.

with butanolic HCl (60C, 15 min), followed by evaporation to dryness, and reconstitution in 100 μl 2% acetonitrile in water.

For measurements in cell culture medium, 50 μl sample was added to 50 μl IS. After subsequent addition of 500 μl acetonitrile, samples were centrifuged (10 min, 12.000 × *g*), the supernatant was evaporated to dryness (N_2, 40 C), and derivatized with butanolic HCl (60 C, 15 min). Subsequently, samples were evaporated to dryness and reconstituted in 100 μl 2% acetonitrile in water.

A 10-μl aliquot of the final sample was injected for HPLC-MS/MS analysis.

Instrumentation

HPLC. Chromatographic separation was achieved on a Surveyor modular HPLC System (Thermo Finnigan Corp., San Jose, CA) consisting of a thermostated autosampler, a low-flow quaternary MS pump, and a 20 × 2 mm Supelguard Discovery C8 column (Sigma-Aldrich Chemie B.V. Zwijndrecht, The Netherlands). The sample was eluted at a flow rate of 300 μl/min and a linear gradient of 5 min: start = 97% eluant A (0.1% formic acid in water), 3% eluant B (acetonitrile/water: 9/1); end = 100% eluant B. The system was held on for 3 min with the initial eluant at a flow rate of 500 μl/min to equilibrate the column; the column temperature was maintained at 20 C.

Mass spectrometry. MS/MS analyses were performed on a TSQ Quantum AM mass spectrometer (Thermo Finnigan) using positive electrospray ionization. Nitrogen was used as a sheath and drying gas; argon was used as collision gas at a pressure of 0.20 Pa. The capillary voltage used was 3.5 kV. The source temperature was set at 85 C; optimal cone voltage ranged 30–40 V for the analytes and IS. DIT, MIT, and MCT were measured by multiple reaction monitoring (MRM) using transitions m/z 490 → m/z 388 for DIT, m/z 364 → m/z 135 for MIT, and m/z 272 → m/z 135 for MCT, with an optimal collision energy of 21, 42, and 42 eV, respectively. Settings were optimized using a 0.1-μmol/liter solution of DIT, MIT, and MCT in water and urine.

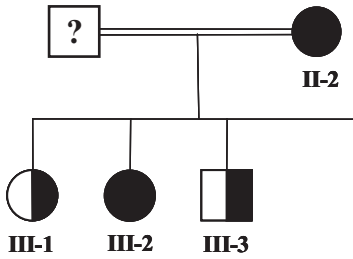
Patients

The index patient (See II-2 in Fig. 3) comes from a highly consanguineous Belgian/Moroccan family. She came to medical attention in 1967 at the age of 5 yr when she presented with delayed psychomotor development, stunted growth, and goiter. This phenotype was clearly family related, with several sibs and grandparents affected with a seemingly recessive mode of inheritance. At that time a loading test with iv administrated radiolabeled DIT showed excretion of 50–70% of the administered dose, in contrast to controls that secreted 15–20%. These data resulted in the diagnosis DD and were supported by the successful treatment of one of the subjects with lugol (15). Patient III-2 was born with a goiter and, subsequently, diagnosed with hypothyroidism due to dyshormonogenesis (TSH: 75 mU/liter; and T_4: 60 nmol/liter). She had prolonged neonatal jaundice due to cholestasis. A liver biopsy confirmed Byler disease, and at the age of 3 yr, she received a liver transplant. At that time the family was approached for detailed testing of their thyroid status, and informed consent for DNA testing was given. Plasma was collected for TSH, T_4, free T_4 (fT_4), T_3, TG, and thyroxine-binding globulin determination. Urine was collected to determine iodine, DIT, and MIT excretion, and peripheral blood cells were harvested for analysis of genomic DNA.

Urinary MIT and DIT excretion was measured in 24 healthy adults from whom material was collected to determine iodine excretion in relation to food status (National Institute for Public Health and the Environment) and 23 healthy schoolchildren of 5 and 6-yr-old who anonymously contributed spot urine to determine a iodine excretion reference range (all with informed consent).

Mutation analysis of IYD

Primers (sequences available upon request) were designed and synthesized (Biolegio) to cover the six coding exons of *IYD*, based on both major *IYD* splice forms (13), by PCR amplification. Using AmpliTaq Gold (Applied Biosystems, Foster City, CA) under standard conditions, PCR fragments were generated from patient blood DNA. Big Dye Terminator V3.1 (Applied Biosystems) nucleotide sequence analysis was performed on the PCR fragments, and the resulting sequences (ABI3100/3730) were compared with the *IYD* reference genomic sequence (GenBank accession no. NT_025741) using CodonCode Aligner (CodonCode Corp., Dedham, MA). cDNA reference nucleotide numbering was performed according to GenBank accession no. NM_203395, and amino acid numbering according to GenBank accession no. NP_981932.



Year of birth		1988	1989	1990	1963	1993	1994	Reference value
Goiter		No	Yes	No	Yes	No	No	No
T4 treatment		No	Yes	No	Yes	No	No	No
TSH (mU/L)	SERUM	nd	0.6	2.1	1.2	0.5	1.1	0.4-4
T4 (nmol/L)		nd	130	140	95	125	110	70-150
fT4 (pmol/L)		nd	15.7	11.9	6.4	13.7	10.8	10-23
T3 (nmol/L)		nd	1.90	2.60	2.95	2.25	2.60	1.30-2.70
TG (pmol/L)		nd	<20	72	1040	110	52	5-60
TBG (nmol/L)		nd	380	500	500	410	430	200-650
Iodine excretion (nmol/L)	URINE	nd	454	320	696	561	525	315-1575
MIT excretion (nmol/L)		nd	4.9	4.2	220.8	100.8	14.1	nd
DIT excretion (nmol/L)		nd	1.4	<1	108.2	31.2	1.7	nd
IYD genotype c.658G>A p.A220T	DNA	G/A	A/A	G/A	A/A	G/A	G/A	G/G

		Reference value
Goiter	Yes	No
TSH (mU/L)	>100	0.7-6.4
fT4 (ng/dl)	<0.3	0.8-1.9
fT3 (pg/ml)	2.60	1.45-4.50
TG (ng/ml)	1887	1-70
Anti-TG (U/ml)	<20	<40
Anti-TPO (U/ml)	<10	<35

FIG. 3. Genotypical and phenotypical data of family with DD. *Top panel* shows data from urine/serum/DNA from DD family members sampled in 2002. *Bottom panel* shows serum levels from patient III-4 sampled in February 2008. TBG, Thyroxine-binding globulin; TPO, thyroid peroxidase.

Plasmids

An *IYD* fragment encompassing nucleotide 138-1010 (GenBank accession no. NM_203395.1) was amplified from normal human thyroid cDNA and cloned into the vector pDONR/ZEO using Gateway Technology (Invitrogen Corp., Carlsbad, CA). IYD-FLAG fragments were generated by PCR using primers 5′-GCCACCATGTATTTCCTGACTCCCATCT-3′ and 5′-AGGCCTACTTATCGTCGTCATCCTTGTAATCCACTGTCACCATGATCTGGT-3, subcloned into pGEM-Teasy (Promega Corp., Madison, WI), and finally ligated into the cytomegalovirus promoter-based expression vector pCDNA3 (Invitrogen). Site-directed mutagenesis using the QuikChangeII protocol (Stratagene, La Jolla, CA) was performed to mutate the alanine on position 220 (GenBank accession NP_981932) into a threonine or a serine.

Cell culture and transfection

HEK-293 cells obtained from the American Type Culture Collection (CRL-1573; Manassas, VA) were maintained in DMEM supplemented with 10% fetal bovine serum (BioWhittaker, Inc., Walkersville, MD). One day before transfection, cells were seeded in a six, 12, or 24-well culture dish. Transfection was performed with Fugene6 transfection reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Transfection efficiency was determined by cotransfection of pEGFP-C1 (Clontech Laboratories, Inc., Mountain View, CA). The percentage of fluorescent cells was determined using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). IYD-FLAG protein expression was determined by Western blot analysis (FLAG M2 antibody; Stratagene) on cell lysates.

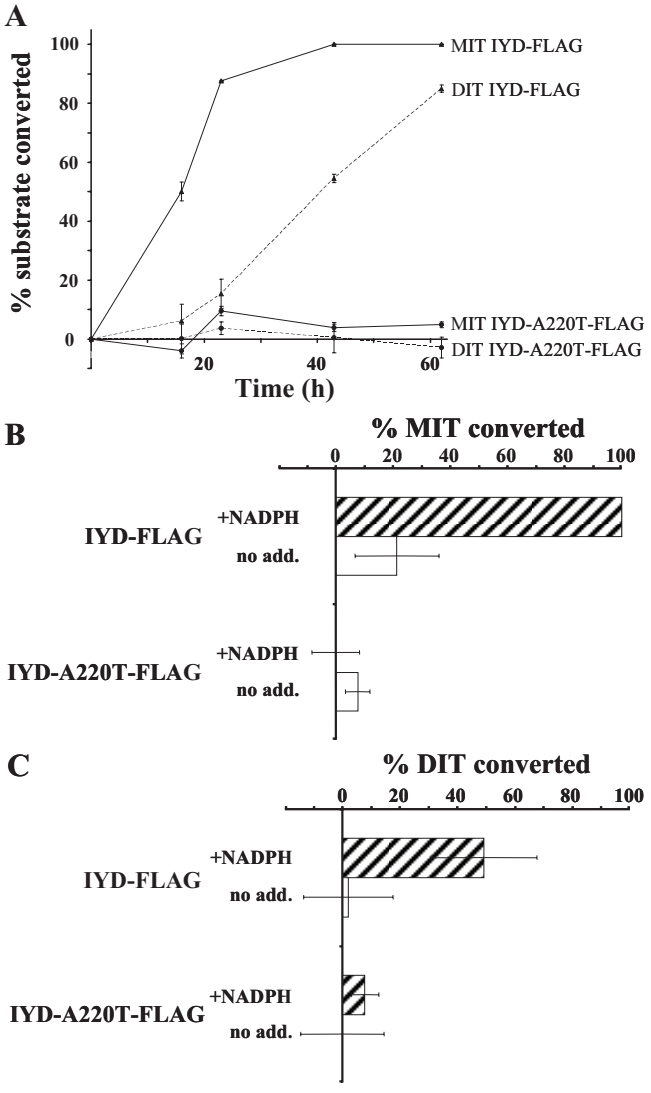


FIG. 4. The IYD mutation of alanine-220 into a threonine abolishes dehalogenase activity. HEK293 cells (six-well format) were transfected with pCDNA3, IYD-FLAG, or IYD-A220T-FLAG DNA. A, Twelve hours after transfection, 0.5 μM MIT or 0.25 μM DIT was added to the cells (total volume 2 ml). At the indicated time points, 50 μl samples were taken, and MIT-DIT concentrations were determined by HPLC-MS/MS. Percent substrate converted is relative to cells transfected with pCDNA3. Values are averages from duplicate samples ± SD. B–C, Two days after transfection, cells were harvested and lysed. The lysate was incubated for 3 h in assay buffer with or without 0.1 mM NADPH (13) in the presence of either 100 nM MIT (B) or 50 nM DIT (C). After the incubation a 50-μl sample was taken, and MIT-DIT concentrations were determined by HPLC-MS/MS. Percent substrate converted is relative to cells transfected with pCDNA3. Values are averages from duplicate samples ± SD.

Results

Validation HPLC-MS/MS assay

No sizeable background signal or mutual interferences were observed for MIT, DIT, and MCT in the HPLC and MS assay (Fig. 1). In urine, signal responses for MIT and DIT decrease by respectively 1.1% (R = 0.53) and 2.2% (R = 0.63) per nmol/liter creatinine. The limit of detection (LOD) was defined as the lowest signals detected with a S/N of three. The LOD for MIT and DIT in water was 0.2 nmol/liter. Depending on the extent of suppression, LOD in urine was between 0.2 and 2 nmol/liter, and LOD ranged between 0.1 and 0.4 nmol/mmol creatinine for the corresponding creatinine range 0.5–20 mmol/liter. Of the urine sample, 100 μl was needed to determine MIT and DIT with a S/N of more than three. Overall, in urine, MIT could be determined with a coefficient of variation (CV) less than 7% (20–1000 nmol/liter) and a CV less than 15% near LOD; DIT could be determined with a CV less than 15% (20–1000 nmol/liter) and a CV less than 25% near LOD. More details on the validation are available on request.

MIT-DIT determination in patient urine samples

DIT and MIT levels in urine of individuals without thyroid disease were determined by HPLC-MS/MS. In addition, the index patient with a presumed DD and four of her children were included. The urinary MIT-DIT data are graphically presented in Fig. 2. The average values in the group of 57 individuals without any known thyroid disease are 3.2 ± 2.1 nmol/liter MIT (range 1.0–9.8) and 0.6 ± 0.4 nmol/liter DIT (range 0.2–2.7). For a subgroup of 24 adults without any known thyroid disease (average 2.6 ± 1.5 nmol/liter MIT, 0.5 ± 0.1 nmol/liter DIT), we could also calculate the daily MIT-DIT excretion: 1.4 ± 1.2 nmol MIT/d, 0.3 ± 0.1 nmol DIT/d. Two members of the DD family (III-4 and II-2) showed increased levels of both DIT and MIT in their urine.

IYD mutation analysis in individuals with increased urinary MIT-DIT

For six members of the DD family, blood DNA was available for nucleotide sequence analysis of the entire coding area and the exon/intron boundaries of *IYD*. Index patient II-2 of the DD family, as well as her affected daughter (III-2), are homozygous for a missense nucleotide substitution in exon 4 of *IYD* (c.658G>A, p.Ala220Thr). Both had clinical symptoms of hypothyroidism and are currently treated with T_4 supplementation aimed at keeping TSH levels within the reference range. Four additional children were available for genetic analysis, and they are heterozygous for the mutation. At the DNA testing (2002), all were clinically euthyroid and did not have a palpable goiter, despite the clearly elevated serum TG and urine MIT-DIT levels in III-4. In February 2008, patient III-4 came to medical attention with complaints of goiter, weight loss, fatigue, and lack of appetite. Thyroid function test showed increased TSH and TG, decreased T_4 and T_3, without any signs of thyroid autoimmunity. T_4 therapy was initiated immediately.

MIT-DIT levels, *IYD* genotype, and clinical thyroid parameters of the investigated DD family members are summarized in Fig. 3.

The direct sequence analysis of 100 control alleles showed the presence of one *IYD* c.658A allele.

Functional analysis of IYD and the IYD A220T mutation

The A220T mutation falls within the enzymatic dehalogenase domain of IYD (11–13). To assess whether the IYD-A220T mutation has functional consequences, HEK293 cells were transfected with different cytomegalovirus promoter-IYD expression plasmids. IYD activity is measured by the decrease of substrate (MIT or DIT) in tissue culture medium as determined by HPLC-MS/MS. Expression of wild-type IYD-FLAG protein in a 24-well

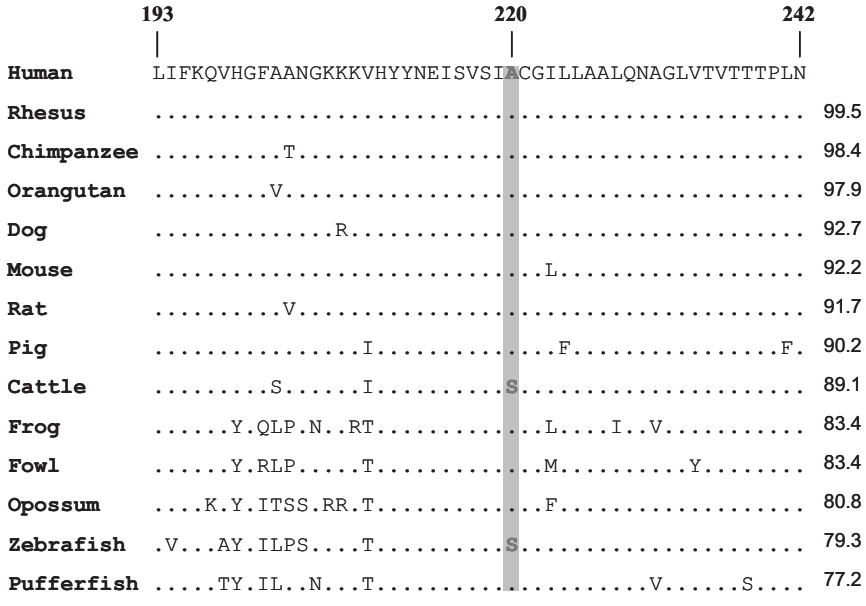


FIG. 5. IYD is strongly conserved in vertebrates. The iodotyrosine dehalogenase domain protein sequences of the rhesus monkey (GenBank accession no. XP_001099028), chimpanzee (GenBank accession no. XP_527537), orangutan (Swissprot Q5REW1), dog (GenBank accession no. XP_533449), mouse (GenBank accession no. NP_081667), rat (Swissprot Q5BK17), pig (Swissprot Q6TA49), cattle (GenBank accession no. XP_869498), frog (GenBank accession no. NP_001087329), fowl (GenBank accession no. XP_419670), opossum (GenBank accession no. XP_001381189), zebrafish (GenBank accession no. XP_001335518), and pufferfish (GenBank accession no. CR693638) were obtained by Basic Local Alignment Search Tool search with the human iodotyrosine dehalogenase domain (GenBank accession no. NP_981932, amino acid 93–285). Multiple alignments were performed using ClustalW (European Bioinformatics Institute, Cambridge, UK). Part of the iodotyrosine dehalogenase domain that ranges from amino acid 193–242 is shown (amino acid numbering according to the human sequence). Identical amino acids are shown as *dots*. The *shaded area* indicates the amino acid corresponding to human alanine-220. At the end of each sequence, the percentage of identical amino acids with the complete human iodotyrosine dehalogenase domain is depicted.

format shows a complete conversion of MIT within 43 h after addition of the substrate. The conversion of DIT is slower, but after 62 h, over 80% of DIT is converted. IYD activity is not affected by the addition of the FLAG tag, and can be completely inhibited by the IYD inhibitor 3-nitro-L-tyrosine (16) (data not shown). The IYD-A220T mutant does not result in significant MIT or DIT conversion (Fig. 4A).

To exclude that the difference in dehalogenation kinetics between MIT and DIT is the result of differences in substrate membrane transport, the IYD enzymatic activity was also measured in lysates of transfected cells. As shown in Fig. 4, B and C, also in lysates MIT is more rapidly converted than DIT upon addition of nicotinamide adenine dinucleotide phosphate (NADPH). Again, cells transfected with the mutant IYD-A220T do not display any dehalogenase activity.

The enzymatic dehalogenase domain of IYD is strongly conserved among vertebrates. Alignment shows that even in the most distantly related vertebrates to human, the zebrafish and pufferfish, there is a more that 77% identity at the amino acid level (Fig. 5). Alanine-220 is also highly conserved among the vertebrates listed. Only cattle and zebrafish have a variant serine at this position. Functional comparison of the alanine, threonine, and serine at position 220 shows that both the alanine and serine-containing IYD have full enzymatic activity, whereas cells transfected with IYD-A220T lack dehalogenase activity (Fig. 6A).

Western blot analysis indicates that the amount of IYD protein in cells transfected with IYD-A220T DNA is much less than in cells transfected with similar amounts of DNA containing either an alanine or serine at position 220 (Fig. 6A). To exclude that the lack of dehalogenase activity of the IYD-A220T mutant is completely the result of diminished protein production, cells were transfected with a decreased amount of IYD-FLAG plasmid. As shown in Fig. 6B, even when expressing substantial higher amounts of mutant protein compared with wild-type protein, the mutant IYD-A220T protein still does not display any enzymatic activity.

Discussion

MIT and DIT determination using HPLC-MS/MS

In the 1950s and 1960s, patients suffering from primary hypothyroidism often developed goiter requiring thyroidectomy, and the availability of goitrous thyroid tissue made direct measurement of dehalogenase enzyme activity possible. In addition, the measurement of dehalogenase activity by measuring DIT conversion after *in vivo* application of radiolabeled DIT was used for diagnostic purposes, a method currently not advocated. Although progress has been made in the development of assays to detect DIT and MIT (17–20), most methods lack selectivity and/or are too laborious and time consuming to qualify as high-throughput screening methods. One of the current diagnostic obstacles to identify DD patients at an early stage is a proper tool to measure increased levels of DIT and MIT in urine or blood, one of the hallmarks of DD (6). Iodotyrosine detection in urine by mass spectrometry will facilitate the neonatal detection of DD (14). This early detection followed up by early treatment might prevent the mental retardation resulting from thyroid hormone deficiency in early life observed by us and others (14) in patients suffering from DD.

In this paper we describe a rapid, highly selective, and sensitive HPLC-MS/MS assay to determine MIT and DIT levels in urine. In normal subjects, urinary DIT levels of 1.23 nmol/24 h were determined by RIA (21), or 0.76 nmol/24 h as determined by a gas chromatographic mass spectrometric assay (22), which are levels in the same order of magnitude as our data of 0.3 ± 0.1 nmol DIT/d in adults without any known thyroid disease. In this group the daily MIT excretion is 1.4 ± 1.2 nmol MIT/d. As far as we know, MIT levels in urine have never been determined before.

Structure-function analysis of recombinant IYD protein

Although *in vitro* expression studies show that the IYD-A220T mutation results in reduced protein expression, the functional consequence of the alanine to threonine substitution is a

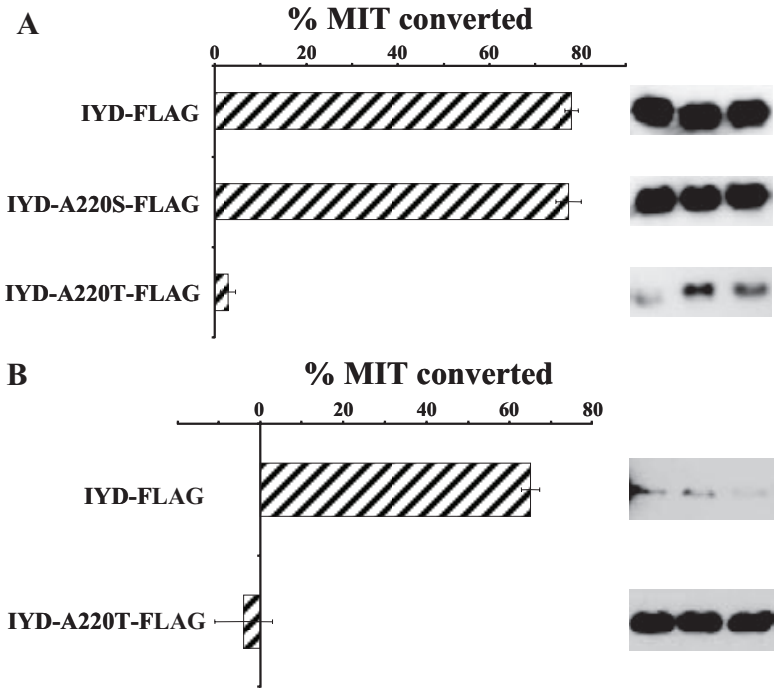


FIG. 6. Threonine at position 220 results in a decrease of protein production and inactivation of enzyme activity. HEK-293 cells (A: 24-well format; B: 12-well format) were transfected with the indicated expression vectors. A, Twenty-two hours after transfection, MIT was added to a final concentration of 1 μM. Twenty-four hours later, a 50-μl sample was taken for MIT determination. B, The amount of IYD-FLAG DNA used for transfection is one tenth the amount of IYD-A220T-FLAG. Seven hours after transfection, MIT was added to the 1 ml/well culture medium to a final concentration of 1 μM. Thirty-nine hours after the addition, a 50-μl sample was taken for MIT determination. Percent MIT converted is relative to cells transfected with pCDNA3. Values are mean ± SD of triplicate samples. Transfection efficiencies as determined by cotransfection of pEGFP-C1 DNA and flow cytometry were: IYD-FLAG 32%, IYD-A220T-FLAG 27%, and IYD-A220S-FLAG 32% (A); and IYD-FLAG 15% and IYD-A220T-FLAG 24% (B). On the *right side* of the graph, the corresponding Western blot signals are shown with the FLAG M2 antibody.

complete abrogation of the enzymatic dehalogenase activity. This reduced *in vitro* protein expression has also been observed for an IYD-I116Y mutant that was reported, in contrast to two other mutations within the dehalogenase domain that apparently only affect the enzymatic activity (14). Strikingly, it is not so much the location of the mutation as the nature of the amino acid change because the *in vitro* production of the IYD-A220S is comparable to the wild-type protein.

The alanine-220 residue is highly conserved in the vast majority of all currently known vertebrate IYD protein sequences (and also in more distantly related eukaryotes; data not shown). Cattle and zebrafish have a serine at this position, but we showed that this has no effect on IYD enzymatic activity. Based on the structural homology of the iodotyrosine dehalogenases with the bacterial NADH oxidase/flavin reductases, alanine-220 might be involved in flavin mononucleotide binding (12), whereas according to the conserved domains database (23), the area surrounding position 220 is the putative dimer interface.

It remains to be shown whether the loss of enzymatic activity, reduced protein expression, or a combination is responsible for the dehalogenation defect *in vivo*.

Phenotype-genotype correlation within a family with an inactivating IYD mutation

The index patient (II-2), who presented with clinical hypothyroidism (delayed psychomotor development, stunted growth, and goiter) at the age of 5 yr, is homozygous for the inactivating IYD-A220T mutation. The hypothyroid/goitrous phenotype is familial, and many family members suffer from mental retardation (15). We could speculate that this is due because of a chronic iodine deficiency caused by a dehalogenase defect that is not compensated by iodide ingestion in affected mother-sib pairs. Although subject II-2 receives thyroxin treatment, her low fT_4 and high-plasma TG suggest poor compliance, explaining the increased MIT and DIT excretion in urine. The fact that although her fT_4 is below the reference range, her T_3 is above suggests an activated metabolic system adapted to low T_4 levels, as can be seen in patients with, for example, a TG synthesis defect (24).

The daughter (III-2) is also homozygous for the inactivating IYD mutation. She has no increased urinary MIT-DIT excretion, probably as a result of proper levothyroxin treatment aimed at maintaining serum TSH levels within the reference range. This is corroborated by her unsuppressed plasma TG levels. However, because she has received a transplanted liver from presumably a normal IYD individual, we cannot completely exclude that this liver contributes to the MIT and DIT deiodination, although at the mRNA level, IYD expression in the liver is very low (13).

Our pedigree analysis as depicted in Fig. 3 is in line with the view that DD is a recessive disease (9, 14, 15, 25, 26). Interestingly, subject III-4 (who is heterozygous for the IYD-A220T mutation), in whom thyroid palpation in the past indicated a normal sized gland, recently came again to medical attention because of goiter and clinical and biochemical signs of hypothyroidism. On hindsight, this correlates with the increased serum TG and urinary MIT and DIT excretion; although at the time of these measurements, there were no other signs of hypothyroidism. The reason for the development of goiter within a period of 4 months in this 14-yr-old boy is not known. However, it does point to a possible dominant behavior of the genetic defect with variable penetrance because the other heterozygous sibs have no goiter, increased plasma TG, or increased MIT or DIT excretion. Apart from the mutation in exon 4, analyses of five polymorphic positions covering IYD intron 1 to exon 5 all show heterozygosity, indicating that both alleles of *IYD* in subject III-4 are present (supplemental Fig. 1, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). Dominant inheritance of DD has been suggested in some families before (25). Although this might be explained by allele-specific *IYD* expression, analysis of mRNA by RT-PCR in normal thyroid tissue did not demonstrate allele-specific *IYD* expression (supplemental Fig. 2).

The occurrence of the mutant allele in the normal population (one in 100) suggests that *IYD* c.658G>A is a functional single

nucleotide polymorphism. It is tempting to speculate that it may play a role in the clinical variable response to iodine deficiency. More investigations are essential to substantiate the role of heterozygous inactivating IYD mutations in the development of noncongenital goiter.

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Address all correspondence and requests for reprints to: Dr. G. Afink, Laboratory Pediatric Endocrinology, Room G2-133, Academic Medical Center, University of Amsterdam, P.O. Box 22660, 1100 DD Amsterdam, The Netherlands. E-mail: g.b.afink@amc.uva.nl.

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